EFFECTS OF PARENTHOOD ON NEURAL RESPONSES TO PUP-RELATED SENSORY STIMULI

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ABSTRACT

Few mammalian species exhibit biparental care (i.e., parental care by both mothers and fathers). The onset of parental care in female mammals is associated with plasticity in neural processing of infant-related sensory stimuli, which enhances mothers' ability to detect and care for their offspring; however, little is known about sensory plasticity in fathers. We tested the hypothesis that parenthood alters neural responses to olfactory and auditory stimuli from infants in male California mice (*Peromyscus californicus*), a biparental rodent. Virgin males and new fathers were exposed to a combination of a chemosensory stimulus and an auditory stimulus. The chemosensory stimulus was either pup-scented cotton or unscented cotton (control), while the auditory stimulus was a recording of either pup vocalizations or white noise (control). Brains were collected one hour later and stained immunohistochemically for Fos, an index of neural activity. We quantified Fos in the main olfactory bulbs (MOB), a region essential to perceiving olfactory information, and the medial preoptic area (MPOA), a region critical for parental behavior. We predicted that Fos in the MOB and MPOA would be greater in fathers than in virgins, especially after exposure to pup stimuli. We found that fathers had lower expression of Fos in the MOB but higher expression in the MPOA, compared to virgin males. Moreover, Fos in the MPOA was higher in males exposed to pup vocalizations and pup scent compared to those exposed exclusively to pup vocalizations. Fos in the MPOA was also higher in males exposed to scent or both scent and vocalization stimuli compared to males exposed to control stimuli. These findings suggest that the onset of parenthood alters activity in the MOB and MPOA, especially in response to pup vocalizations and scents, in males in the biparental California mouse.

Keywords: audition, biparental care, brain, California mouse, neural plasticity, olfaction, paternal behavior

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INTRODUCTION

Maternal care, or the care of young by their mother, is essential for offspring survival in all mammalian species. The onset of maternal care is associated with neural plasticity in the mother's brain, mediated by hormonal changes that mothers experience during pregnancy, parturition and lactation, including changes in estrogen, progesterone, prolactin, and oxytocin (Leuner & Sabihi, 2016). Neural plasticity refers to the reorganization of neural pathways in the brain and can include changes in the production, survival, morphology, and activity of neurons and synapses. These structural and functional changes in the brains of new mothers can enable mothers to behave appropriately toward their offspring.

Paternal care – i.e., the care of offspring by fathers – is also important for offspring survival and development in some mammals, including humans; however, relatively little is known about the neural mechanisms underlying the onset of paternal behavior. Fathers in biparental species undergo changes in the activity and structure of neurons, as well as in levels of hormones, neuropeptides, and their receptors, in several brain regions; however, it is probable that there are many additional neural changes associated with the onset of paternal care that have yet to be discovered (Horrell et al., 2021).

One type of neural plasticity associated with the onset of offspring care by mothers is sensory plasticity, which involves changes in neural pathways involved in detecting and processing sensory stimuli. For example, in CBA/CaJ house mice (*Mus musculus*), new mothers undergo changes in the brain's auditory pathway that enhance their ability to detect pup vocalizations (Dunlap et al., 2020). In the same species, Liu et al. (2006) found that mothers had higher neural entrainment, or neural responses quantified by spike activity, in response to

auditory stimuli within the frequency range of pup calls, compared to virgin females; this supports the statement that house mouse mothers experience neural plasticity in their responses to pup auditory stimuli. Ehret et al. (1987) also found that house mouse mothers and virgin females with 5 days of repeated pup exposure showed behavioral responsiveness and initiated pup-retrieval behavior in response to sounds within pup-call frequency, whereas virgin females with no pup exposure did not respond. In addition, during the onset of motherhood, some mammals, including house mice, have a significant increase in neurogenesis (i.e., production of new neurons) in the olfactory bulbs, the first brain region that receives information about olfactory stimuli (Medina & Workman, 2018).

Some evidence suggests that fathers, too, undergo plasticity in sensory systems. For example, increased neurogenesis in the olfactory bulbs has been found in C57BL6 house mouse fathers (Mak & Weiss, 2010), similar to mothers. However, sensory plasticity in fathers has received very little attention, especially in biparental species. Characterizing this plasticity in fathers and elucidating the underlying neural and neuroendocrine mechanisms would enhance our understanding of both the effects of fatherhood on the brain and, conversely, the neural processes underlying the onset of paternal care.

This study investigated the neural pathways that are activated in response to pup-related sensory stimuli – pup scents and vocalizations – in male California mice, as well as the effects of fatherhood on these neural responses. California mice (*Peromyscus californicus*) are one of the few mammals that are biparental, meaning that both parents provide care for their offspring (Gubernick et al., 1987); thus, they are a useful model for examining the neural mechanisms underlying the onset of parental care in both sexes. Both mothers and fathers in this species are strongly attracted to their offspring and begin to huddle, lick, and carry pups shortly after

parturition. Parents are also strongly attracted to and nurturant toward unrelated pups (de Jong et al., 2009; Perea-Rodriguez et al., 2015; Horrell et al., 2017; Perea-Rodriguez et al., 2018). In contrast, virgin adult males and females often avoid or attack experimentally presented pups (Gubernick et al., 1994; de Jong et al., 2009; Horrell et al., 2017; Nguyen et al., 2020).

Neural pathways involved in olfactory and auditory processing interact with pathways involved in the onset of parental care, suggesting that olfaction and audition may be important sensory modalities for parental care, at least in rodents (Kuroda et al., 2011; Horrell et al., 2019; Numan, 2020; Wilson et al., 2022). The main olfactory bulbs (MOB) are essential for the detection of chemosensory stimuli and may have downstream effects on parental behavior by sending information to other brain regions. One such region is the medial preoptic area of the hypothalamus (MPOA), which is crucial for parental behavior in both males and females (Numan et al., 2005; Akther et al., 2014; Bales & Saltzman, 2016; Horrell et al., 2019). In biparental California mice, Lee and Brown (2002) performed electrolytic or sham lesion in the MPOA of male and female California mice and found that lesioning significantly reduced parental behavior. The MPOA also receives information from the auditory system, suggesting that sounds, in addition to scents, can influence parental care. Moreover, rodent pup ultrasonic vocalizations have been characterized as having unique properties including situation-dependent frequency and duration eliciting maternal behavior (Shair et al., 2018).

In addition to the published research investigating the role of olfaction and audition in rodent parental care, evidence suggests that multisensory integration may be involved in the perception and integration of both senses combined. Multisensory integration refers to the integration of simultaneously presented sensory stimuli of unique modalities (e.g. auditory and olfactory) into a single multisensory signal (Marks et al., 2018). Okabe et al. (2013) exposed

C57BL/6 house mouse new mothers to various combinations of the following stimuli: hypothermic pups, pre-recorded ultrasonic pup vocalizations, pup-scented cotton, and no stimulus. They then analyzed neural activation via immunohistochemistry for Fos. Fos is the protein product of the immediate early gene c-fos; quantification of Fos can be used as an indicator of neural activation in brain regions of interest. They found significantly higher Fos expression in the MOB and MPOA of mothers exposed to ultrasonic pup vocalizations and pup odor cotton simultaneously as opposed to those exposed to no stimulus, suggesting that there is multisensory integration of olfactory and auditory sensory information during the onset of motherhood in house mice.

In this study, we tested the hypotheses that the MOB and MPOA of male California mice have greater neural responses to pup sensory stimuli compared to control stimuli, that fathers have greater neural responses to pup sensory stimuli compared to virgin males, and that fathers exposed simultaneously to auditory and olfactory stimuli have greater neural responses than those exposed to a single stimulus.

METHODS

Animals

All experimental procedures and laboratory conditions were approved by UCR's Institutional Animal Care and Use Committee. Subjects were descended from California mice that were purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA) and bred at the University of California, Riverside (UCR). Mice were housed in polycarbonate cages (44 × 24 × 20 cm) with aspen shavings for bedding, cotton for nesting, and ad libitum access to food (Purina 5001 Rodent Chow) and water. The mice were maintained in

standard laboratory conditions, including temperature of approximately 23°C, 65% humidity, and a 14:10 h cycle. Cages were checked twice daily, and bedding was changed weekly. At weaning age (27–31 days), before the birth of younger siblings, animals were removed from their parents' cages and housed in groups of 2-4 same-sex, age-matched mice until used in the study.

We used 32 breeding pairs housed with their first litter of pups and 29 virgin pairs consisting of a reproductively inexperienced male and an ovariectomized female. Ovariectomies (i.e., surgical removal of both ovaries), as described by Zhao et al., 2018; Andrew et al., 2019, were performed 10 days prior to pair formation. Females were anesthetized with isoflurane, their abdomens were shaved and a midline incision of approximately 1cm in length was made. In females assigned to the ovariectomy procedure (virgin female), both the left and right ovaries were located then cut with microscissors. In females assigned to the sham procedure (breeding female), both ovaries were located and returned to the body cavity. Then, the abdominal muscle was sutured and the skin was closed with tissue glue. All females were treated with pain medication and isolated for 7 days to recover from the procedure.

Stimulus Exposure

Each adult male mouse underwent a single stimulus-exposure test (Table 1). At the beginning of the test, the mouse was removed from its home cage and placed individually in a clean cage (12 x 7.5 x 5.25 cm) with bedding, food, and water, located in a sound-attenuated room; testing the animals in clean cages controls for exposure to pup or mate sensory stimuli from the bedding. The mouse was undisturbed for 110 minutes to allow it to acclimate to the cage and for any Fos activity in their brains related to home cage experiences or handling to dissipate. An auditory stimulus and a chemosensory stimulus were then presented simultaneously for 10 minutes. The

auditory stimulus consisted of either pre-recorded vocalizations from an unrelated pup or white noise (control), and the chemosensory stimulus was either cotton containing the scent of an unrelated pup or fresh cotton (control); the cotton was contained in a wire mesh tea ball (Ø: 8 cm) to prevent the mouse from handling it. To obtain the pup scent, an unrelated 3- to 7-day old pup was wiped with a cotton ball 30 times across its ventrum and anogenital region. To obtain and pre-record the pup vocalization, an isolated 4-day-old and unrelated pup was recorded using a BAT miniMIC (Binary Acoustic Technology, Tucson, AZ; USA) and Spectr III software (Spectral Analysis, Digital Tuning, and Recording Software; Binary Acoustic Technology, Tucson, AZ; USA). During the test, the recording was played via speaker underneath the cage on a 25-s loop (Wilson et al., 2022). Tests were conducted at 08:00-09:00 h. We performed tests during lights-on, which is the inactive period for this nocturnal species, to reduce the amount of background neural activity. Fathers were tested 4-6 days after the birth of their first litter, and virgins were tested at a comparable age and time point.

Table 1. Experimental design

Pup Stimulus	Stimulus Pair	Male Sample Size
Pup Scent	White noise + **Pup cotton**	8 fathers, 8 virgin males
Pup Call	**Pup calls** + Fresh cotton	8 fathers, 6 virgin males
Pup Call + Pup Scent	**Pup calls + Pup cotton**	8 fathers, 8 virgin males
Control	White noise + Fresh cotton	8 fathers, 7 virgin males

Brain Collection and Immunohistochemistry

To determine neural responses to stimuli, we quantified expression of Fos, the protein product of the immediate early gene c-fos, in the MOB and MPOA of male subjects. c-fos and other

immediate early genes are expressed in neurons during genomic activation, beginning approximately 1 hour after exposure to a stimulus, and their protein products can be used as indicators of neural activity (Kovács, 2008).

One hour after the end of the 10-minute stimulus exposure, mice were deeply anesthetized with pentobarbital and euthanized via transcardial perfusion with cold phosphate-buffered saline (PBS 0.1M) and paraformaldehyde (PFA) (de Jong et al., 2009). Brains were harvested and stored for 48 hours in 4% PFA at 4°C. The tissue was then moved to a 30% sucrose solution until fully saturated, then cryoprotected and stored at -20°C. Two to five days prior to slicing, the brains were defrosted and transferred into sucrose solution.

Brains were sliced into 40 um-thick coronal sections using a Leica CM1950 cryostat (Leica Biosystems, Deer Park, IL, USA). Sections were stained immunohistochemically for Fos using rabbit c-Fos antibody overnight followed by goat anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific, Waltham, MA, USA) as the second antibody, allowing for visualization of Fos-positive cells as green in color. All brain sections were incubated in Alexa Fluor 555 for 90 minutes and were exposed to minimal light throughout the remaining procedures. Stained tissues were mounted onto slides, covered and stored for 16 to 22 h at 4 °C, and then and imaged with a Zeiss 880 inverted confocal microscope (Carl Zeiss Microscopy, LLC, White Plains, NY, USA). Lastly, a researcher blind to the parenthood group and stimulus treatment quantified the number of Fos-positive cells using QuPath software (Bankhead et al., 2017). Fos-positive cells were counted in a square section with an area of 200 x 200 μm^2 within the MOB and MPOA regions of interest.

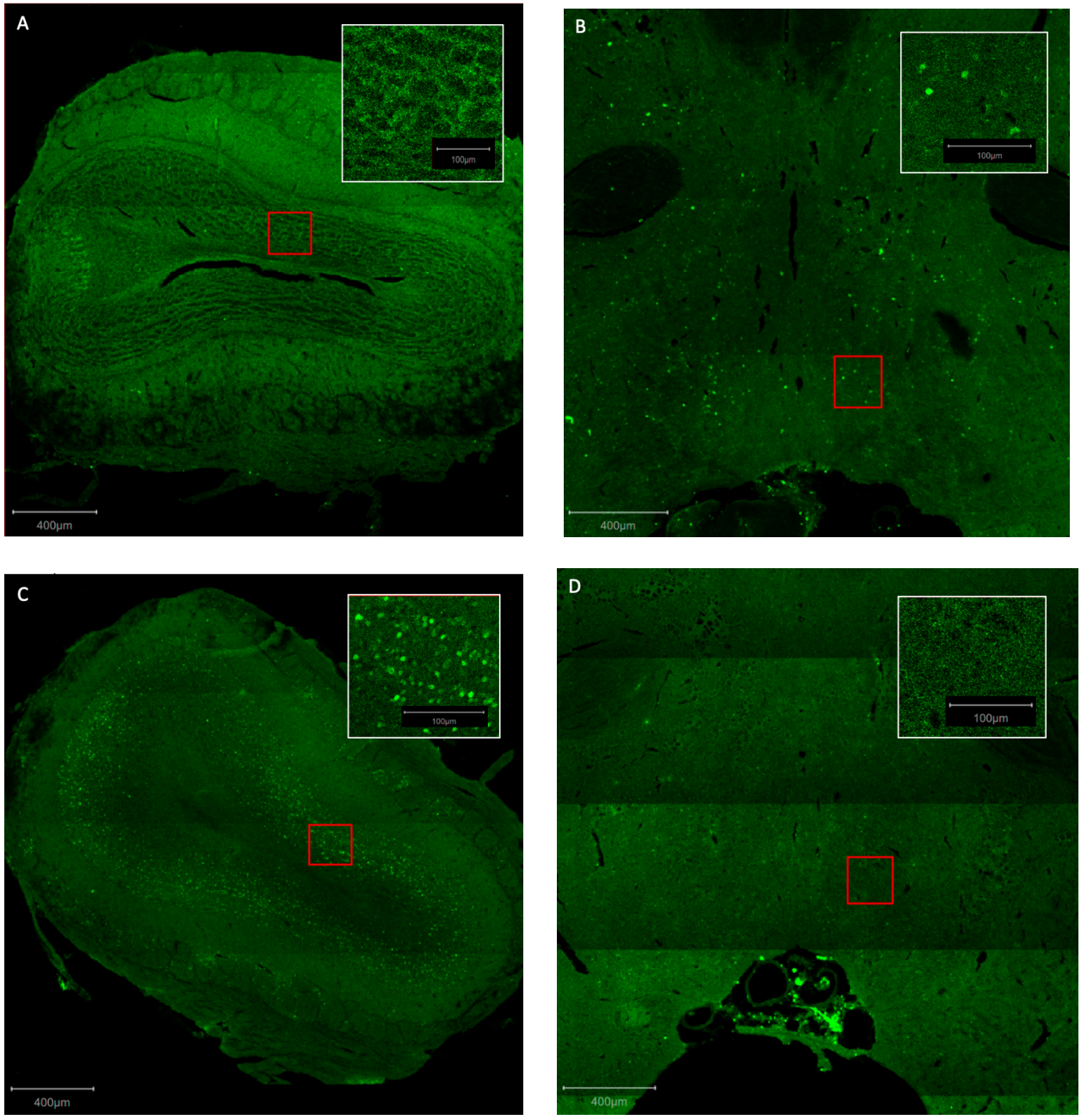

Figure 1. Representative photomicrographs of coronal brain sections showing Fos-positive neurons (bright green dots) in the main olfactory bulbs (A and C) and medial preoptic area (B and D) of California mouse fathers (A, B) and virgin males (C, D). Magnified images are of the area outlined by the red box (200 x 200 µm) in each photomicrograph.

Statistical Analysis

Numbers of Fos-positive cells in each brain region were square-root transformed and analyzed by linear mixed-effect models using STATA 15 (StataCorp LP, College Station, TX, USA). The model for each brain region included reproductive status, stimulus treatment, and their interaction. Significant effects and interactions were further investigated using pairwise post-hoc comparisons.

RESULTS

Main Olfactory Bulbs

Representative photomicrographs of Fos staining the MOB and MPOA are shown in Fig. 1. Reproductive status influenced Fos expression in the MOB (LMM, model: $\chi 2 = 14.76$, $P = 0.005$): Fos in the MOB was significantly higher in virgin males than in fathers (effect of reproductive status: $\chi 2 = 13.44$, $P = 0.0002$). However, no significant difference was found among stimulus treatments ($\chi 2 = 1.14$, $P = 0.768$) (Fig. 2). We did not find a significant interaction between reproductive status and stimulus treatment; thus, the interaction was removed from the final model.

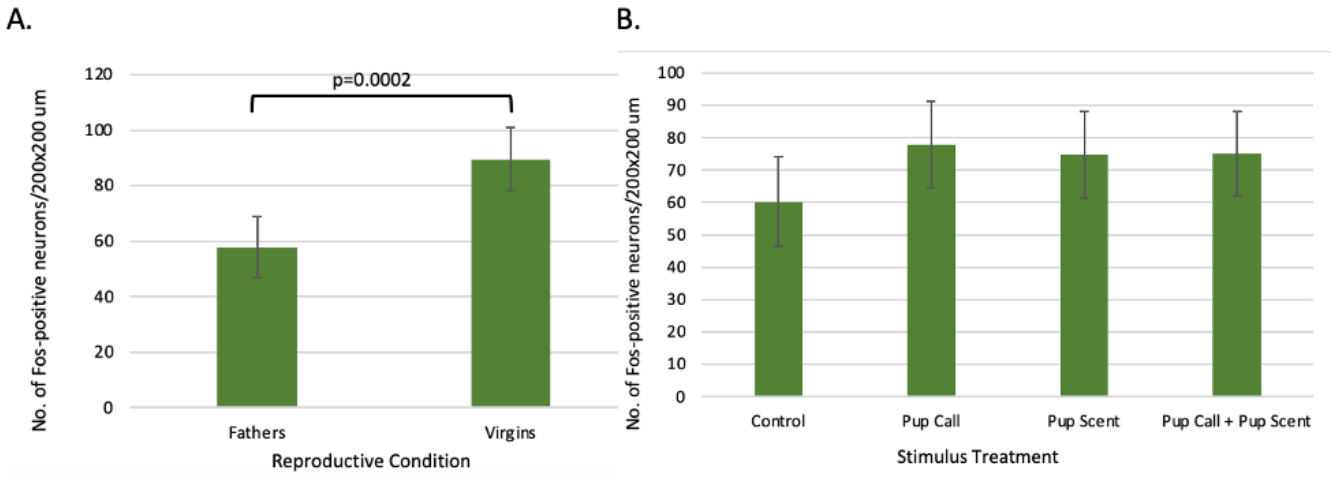

Figure 2. Number of Fos-positive cells (mean ± SE, non-transformed) in the MOB of male California mice. A: Comparison of fathers (N= 30) and virgin males (N=29) collapsed across the four stimulus treatments. B: Comparison across stimulus treatments for fathers and virgins combined (N=14 Control, 14 Pup Call, 14 Pup Scent, 17 Pup Call + Pup Scent).

Medial Preoptic Area

Reproductive status influenced Fos expression in the MPOA (model: $\chi 2 = 118.74$, $P < 0.0001$); Fos was significantly higher in fathers than in virgin males (effect of reproductive status: $\chi 2 = 108.70$, $P<0.0001$; Fig. 3). Moreover, for fathers and virgin males combined, MPOA Fos differed significantly among treatments (effect of treatment: $\chi 2 = 11.32$, $P = 0.010$). Fos expression was significantly higher in males exposed to pup scent only or to both pup calls and pup scent, as compared to males exposed to only the control stimuli (P's < 0.05). Additionally, Fos expression was higher in males exposed to both stimuli than in those exposed only to pup calls (Fig. 3). There was not a significant interaction between reproductive status and stimulus treatment; thus, the interaction was removed from the final model.

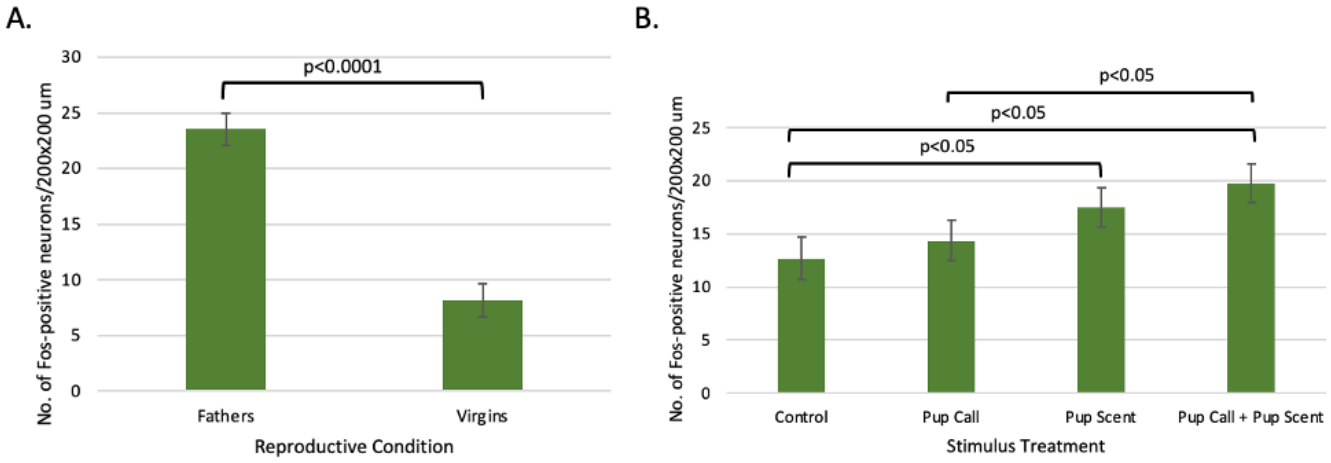

Figure 3. Number of Fos-positive cells (mean ± SE, non-transformed) in the MPOA of male

California mice. A: Comparison of fathers (N= 32) and virgin males (N=29) collapsed across the four stimulus treatments. B: Comparison across stimulus treatments for fathers and virgins combined (N=15 Control, 14 Pup Call, 16 Pup Scent, 16 Pup Call + Pup Scent).

DISCUSSION

The mechanisms underlying the onset of male parental care in biparental mammals are not well understood. Among many unresolved questions, little is known about the role of sensory plasticity in the onset of paternal behavior. In this study, we show that in male California mice, Fos expression is influenced by both reproductive status (MOB and MPOA) and stimulus treatment (MPOA only).

Main Olfactory Bulbs

We hypothesized that neural activity in the MOB would be higher in fathers than in virgin males and higher in males exposed simultaneously to pup scents and pup calls than in those exposed to either pup stimulus alone or to control stimuli only. In contrast, we found that Fos expression in the MOB was lower in fathers than in virgins. Moreover, MOB Fos expression did not differ among stimulus treatments for fathers and virgins combined.

The rodent olfactory system is comprised of a main olfactory system, which is involved primarily in the detection of volatile chemicals, and an accessory olfactory system, which is involved mainly in the detection of non-volatile chemicals such as pheromones. As part of the main olfactory system, volatile odorants enter the nose and are detected by receptors in the main olfactory epithelium. Information about the odorant then travels to the MOB, where the information is further processed and sent to the amygdala. In turn, the amygdala has downstream

effects on the bed nucleus of the stria terminalis (BNST), MPOA, and other regions involved in parental care and other behavior. As part of the accessory olfactory system, non-volatile chemicals such as pheromones enter the nose and are detected by receptors in the vomeronasal organ. Information from the vomeronasal organ is sent to the accessory olfactory bulbs (AOB) and then to the amygdala. Similar to the main olfactory system, the accessory olfactory system has downstream effects on the BNST, MPOA, and other regions (Rymer, 2020).

Previous studies have investigated the role of the olfactory bulbs, which include both the MOB and AOB, in infant-directed behaviors and neurogenesis in male rodents. Kirkpatrick et al. (1994) found that in the biparental prairie vole (*Microtus ochrogaster*), males that underwent removal of the olfactory bulbs attacked pups more frequently than did control males, suggesting that the olfactory bulbs are important for inhibiting aggression towards pups. Mak & Weiss (2010) investigated neural plasticity in house mouse fathers and found that neurogenesis in the olfactory bulbs plays a role in offspring recognition. Although these studies implicate the olfactory bulbs in the expression of pup-directed behavior, we know of no published studies investigating neural responses to pup stimuli specifically in the MOB of males. Our findings suggest that MOB activation is influenced by the onset of fatherhood; however, MOB activation may not be directly influenced by acute exposure to pup sensory stimuli, at least in the California mouse. It is possible that in this species, fatherhood dampens baseline activity of the main olfactory system and that pup odors are detected primarily by the accessory olfactory system.

Medial Preoptic Area

In the MPOA, as predicted, Fos expression was higher in fathers than in virgin males. This finding aligns with a previous study in our lab that found higher Fos expression in the MPOA of

California mouse fathers compared to virgins when males were exposed to a pup (de Jong et al., 2009; but see Horrell et al., 2017). Lambert et al. (2013) also found that in both the California mouse and the uniparental deer mouse (*Peromyscus maniculatus*), fathers exposed to a pup in distress had higher Fos expression in the MPOA than virgin males. Because fathers in our study had higher MPOA Fos expression than virgin males in all stimulus treatments, including the control treatment, our results suggest that activity in the MPOA, a region critical for parental behavior in both sexes, is up-regulated by fatherhood and that this effect is not dependent on acute exposure to pup stimuli.

For fathers and virgin males combined, Fos expression in the MPOA differed among stimulus treatments. Males exposed to either pup scent or pup calls and pup scent combined had higher neural activation in the MPOA than males exposed to control stimuli. In addition, males exposed to pup calls and pup scent combined had higher MPOA Fos than those exposed only to pup calls. These findings suggest that a combination of both olfactory and auditory stimuli from pups may result in additive or synergistic effects on neuronal activation in the MPOA, but that pup scent is a more potent stimulus than pup calls. Similar results have been found in the MPOA of house mouse mothers: mothers had higher neural activity when exposed to pup vocalizations and pup scents simultaneously compared to mothers exposed to pup vocalizations or pup scents alone, suggesting that auditory and olfactory stimuli have synergistic effects (Okabe et al., 2013).

The MPOA is often considered the most essential brain region for parental care (Kohl et al., 2018; Numan, 2020; Rymer, 2020). Numerous studies have investigated the role of the MPOA in activating maternal behavior, including the hormonal influences and neural circuitry mediating this role. In the uniparental rat (*Rattus norvegicus*), new mothers that underwent bilateral electrolytic lesions of the MPOA did not participate in maternal behaviors such as nest

building or retrieval behavior (Jacobson et al., 1980). In addition, Lee & Brown (2002) recorded parental behavior of new fathers and mothers in the California mouse over the first 2 days postpartum. They then performed MPOA electrolytic lesions or sham lesions and again recorded parental behavior. They found that both MPOA-lesioned males and females had significantly greater latencies to exhibit parental care and spent less time exhibiting the parental behaviors recorded, with the exception of huddling. Lee & Brown's (2002) results support the idea that the MPOA is essential for parental behaviors such as sniffing and licking pups. Together, these findings from rats and California mice suggest that the MPOA may be involved in mechanisms of paternal care similar to those involved in maternal care.

The MPOA receives direct input from the amygdala and BNST as well as indirect input from the MOB, AOB, auditory cortex, and somatosensory cortex. Among other regions, the MPOA directly or indirectly interacts with the brain's reward circuitry to inhibit the activity of the nucleus accumbens; this inhibition is thought to increase parental motivation in mothers and, potentially, fathers (Kohl et al., 2018; Numan, 2020). Numan & Numan (1997) utilized a double-labeling immunohistochemical procedure for Fos and wheat germ agglutin in mother rats to identify brain regions that neurons from the MPOA project to when involved in maternal behavior. The double-labeling procedure allowed the investigators to analyze neural activation in addition to retrograde tracing of the neurons involved in signaling. In this experiment, new mothers that exhibited maternal behavior when tested and allowed to interact with pups had higher Fos in the MPOA than females that did not exhibit maternal behavior. They also found that neurons from the MPOA projected most strongly to the medial hypothalamus and lateral septum suggesting that these regions are implicated in maternal behavior. Stack et al. (2001) also found that mother rats had higher Fos expression during maternal behavior. In addition, they

found that Fos-expressing neurons in the MPOA influence the shell of the nucleus accumbens (NAc) suggesting that neurons in the MPOA modulate neural activity in the NAc during maternal behavior.

The MPOA is also an important site of action for hormones that activate parental care. In male and female rats, for example, Rosenblatt & Ceus (1998) found that estradiol implantation in the MPOA resulted in a significantly lower latency to exhibit parental care, suggesting that estradiol in the MPOA mediates parental care in rodent mothers and fathers.

Conclusions

In summary, we found that the onset of fatherhood alters activity of the MOB and MPOA and that pup chemosensory and auditory stimuli, especially when presented simultaneously, alter activation in the MPOA. Our study might have benefited from a larger sample size in order to increase statistical power. In addition, incorporating females would have allowed us to directly compare Fos expression in males and females. Nonetheless, our findings provide novel insights into plasticity in neural responses to pup sensory stimuli during the onset of parenthood and, potentially, into the neural and sensory mechanisms underlying paternal care. Specifically, activation in the MOB and MPOA is altered by the onset of fatherhood, and pup olfactory and auditory stimuli seem to have additive effects on activation in the MPOA.

Further research investigating the responsiveness of other brain regions implicated in receiving and processing sensory information as well as other brain regions involved in parental behavior, such as the BNST, nucleus accumbens, and basolateral nucleus of the amygdala, in response to pup sensory stimuli would contribute to our understanding of male parenting. Moreover, future studies on the responsiveness of the MOB and MPOA to repeated exposure to

pup sensory stimuli, as well as on the cellular and molecular mechanisms of sensory plasticity, would provide valuable insight into potential mechanisms underlying the onset of paternal care and the role of the MOB and MPOA in male biparental rodents.

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